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REVIEW ARTICLE

Human-relevant approaches to assess eye corrosion/irritation potential of agrochemical formulations

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ABSTRACT

There are multiple *in vitro* and *ex vivo* eye irritation and corrosion test methods that are available as internationally harmonized test guidelines for regulatory use. Despite their demonstrated usefulness to a broad range of substances through inter-laboratory validation studies, they have not been widely adopted for testing agrochemical formulations due to a lack of concordance with parallel results from the traditional regulatory test method for this endpoint, the rabbit eye test. The inherent variability of the rabbit test, differences in the anatomy of the rabbit and human eyes, and differences in modelling exposures in rabbit eyes relative to human eyes contribute to this lack of concordance. Ultimately, the regulatory purpose for these tests is protection of human health, and, thus, there is a need for a testing approach based on human biology. This paper reviews the available *in vivo*, *in vitro* and *ex vivo* test methods with respect to their relevance to human ocular anatomy, anticipated exposure scenarios, and the mechanisms of eye irritation/corrosion in humans. Each of the *in vitro* and *ex vivo* methods described is generally appropriate for identifying non-irritants. To discriminate among eye irritants, the human three-dimensional epithelial and full thickness corneal models provide the most detailed information about the severity of irritation. Consideration of the mechanisms of eye irritation, and the strengths and limitations of the *in vivo*, *in vitro* and *ex vivo* test methods, show that the *in vitro/ex vivo* methods are as or more reflective of human biology and less variable than the currently used rabbit approach. Suggestions are made for further optimizing the most promising methods to distinguish between severe (corrosive), moderate, mild and non-irritants and provide information about the reversibility of effects. Also considered is the utility of including additional information (e.g. physical chemical properties), consistent with the Organization for Economic Cooperation and Development's guidance document on an integrated approach to testing and assessment of potential eye irritation. Combining structural and functional information about a test substance with test results from human-relevant methods will ensure the best protection of humans following accidental eye exposure to agrochemicals.

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Introduction

The human eye may be exposed to chemicals through various situations, such as accidental splashing or exposure to chemical particles, vapours or gases. Workers and consumers are usually advised to immediately wash eyes generously with water following exposure. Even without washing, it has been shown that if a substance contacts the surface of the human eye, greater than 80% of it is naturally expelled in less than 2 min [1]. While efficient tear secretion (lachrymation) and drainage pathways help to protect the eye from potentially harmful substances, exposure to chemical substances may lead to irritation or corrosion [2,3]. Therefore, it is essential to be able to categorize the eye irritation potential of industrial and consumer agrochemicals and products so that appropriate hazard statements, handling procedures, protective equipment, and emergency response procedures can be communicated.

Eye irritation tests are conducted on agrochemical active ingredients and formulations to support their registration with regulatory authorities. The traditional regulatory test method for this purpose is the *in vivo* rabbit eye test [4,5]. In the United States (US) alone, the Environmental Protection Agency (EPA) Office of Pesticide Programs receives data on agrochemicals from more than 200 rabbit eye irritation tests each year. However, there is support for moving away from testing on animals, including a recent directive from the EPA Administrator that commits to ending funding of, and requests for, tests on mammals by 2035 and aims to reduce

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animal tests in the shorter term [6]. Eliminating or reducing animal use is also a stated goal of many companies, investor communities, and organizations. As such, there is a strong need to establish a human-relevant, reproducible, reliable, and quantitative alternative approach that can effectively and consistently classify agrochemicals formulations. There are a large number of *in vitro* and *ex vivo* methods, many available as internationally harmonized test guidelines (TGs), which have been developed to identify the eye irritation potential of a wide range of substances. However, their widespread adoption as complete replacements for the *in vivo* rabbit eye test has been hindered by a lack of *in vitro*/*ex vivo* to *in vivo* concordance for specific ranges of eye irritation (in particular distinguishing mild and moderate irritants) and/or specific chemistries. The mechanisms leading to eye irritation/corrosion and the principle and performance of various methods are explored below to identify approaches that can be used to predict human eye irritation potential without the use of *in vivo* testing.

Review of existing data and prospective *in vitro* testing

Agrochemical formulations may be quite complex, typically composed of the active ingredient(s) as well as "inert" ingredients, and both of which may present eye irritation hazards. Accordingly, plausible test methods should be able to address the mechanisms of eye irritation of the active and inert ingredients.

Several publications have evaluated the use of *in vitro* and *ex vivo* methods for assessing the eye irritation/corrosion effects of agrochemical formulations [7–10]. The results demonstrated promise in using these methods but highlighted the need for additional analyses to further understand why *in vitro*/*ex vivo* and *in vivo* rabbit results may not align and to further interrogate the utility of the rabbit test as a reference method for such comparisons. The exaggerated exposure conditions (as described in Table 1) and the anatomical and physiological differences between rabbits and humans call into question the relevance of the *in vivo* response in rabbits to humans. Within the rabbit test, there are multiple points where subjectivity is introduced with respect to observations of reversibility and damage, therefore, results are potentially confounded both by inter-observer variation and animal variation. Further, the likelihood of achieving the same classification upon repeat testing has been shown to be $<$50% for substances which fall into the mild to moderate irritation range [18]. Given the inherent variability of the rabbit test itself, the apparent discordance between the *in vitro*/*ex vivo* methods and the rabbit test could actually be a reflection of uncertainty in the reference classification.

Rethinking the process of establishing confidence in new methods

While hazard categories used by regulatory agencies have been the predominant focus in developing testing approaches, the primary objective is the prediction of human responses, and therefore, a more logical approach would be the identification and optimization of approaches that reliably predict human responses based on faithful representation of relevant human biology. The concept of a 1:1 alignment with the *in vivo* reference classification is neither feasible nor scientifically justified considering the multiple issues associated with the rabbit eye test, which are discussed in Table 1 [18,19]. Accordingly, there is a need to rethink how to assess the validity of new methods and to evaluate test methods based on which are most reliable and relevant to the human response. This paper outlines the available *in vivo*, *in vitro* and *ex vivo* test methods and, considering human ocular anatomy and physiology and mechanisms of chemically induced ocular irritation, their relevance to predicting eye effects in humans following exposure to substances (agrochemicals and formulations), with a goal of identifying those methods most appropriate for human health evaluation.

Structure of the eye across species

Commonly used methods for assessing eye irritation and corrosion are based on the human, rabbit, cow, chicken, or pig eye. To understand which method(s) are most appropriate for studying effects on the human eye, it is important to understand how the structure of the eye differs across species (Figure 1). While comparisons have been made among various species, the preponderance of literature on this topic is focussed on the comparative anatomy and physiology of humans, cows, and rabbits.

There are five layers of the human cornea: the epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium. *Ex vivo* bovine corneas (ca. 850–1000 μm [74]) are thicker than those of pigs (ca. 600–700 μm [75,76]), humans (ca. 550 μm [77,78]), rabbits (ca. 400 μm [77,79]), or chickens (ca. 225–400 μm [80,81]). The thickness of corneal tissue varies depending on the age of animal and method of measurement. Of additional note, corneal thickness can increase after excision from the whole globe or from the conditions of the test method (e.g. from stromal fluid uptake in the *ex vivo* culture system.) Bovine corneal epithelium is thicker than that of humans, pigs, rabbits, or chickens, with 7–9 cells deep in bovine, 4–6 cells deep in humans and pigs, and 4–5 in the rabbit and chicken. In addition to having more cell layers, the cell size may vary between species [82]. Similarly, the bovine corneal stroma is thicker than that of humans and pigs, which in turn is thicker than that of rabbits and chickens. These differences in the thickness of the cornea, and individual corneal layers, can impact the rate of permeation and penetration of chemicals into the cornea (and should be accounted for in test method development). Among the non-human corneas used for eye irritation studies, the porcine cornea is most similar in size and structure to the human cornea. Humans have a Bowman's layer, an acellular collagen-rich zone just under the epithelial basement membrane, which has a unique extracellular matrix organization different from most other species. Bovine, porcine, and rabbit corneas have a functionally analogous layer called the

Table 1. *In vivo*, *ex vivo*, and *in vitro* test methods to assess eye irritation and/or corrosion.

Test	Description	Human relevance: i. Test system ii. Mode of action iii. How the model fits into the DOI concept iv. Exposure time and washing v. Reversibility of tissue lesions
***In vivo* test**		
OECD TG 405/OPPTS 870.2400: Rabbit *In Vivo* Test [5,11]	The test results are used to qualitatively assign ocular irritation scores based on subjective examination of the nature and severity of lesions (including corneal opacity, conjunctivae redness, or swelling of the eyelids) and their reversibility. *Classification:* This test was developed in 1944 [12] and is used to assign one of four EPA categories (I, II, III, or IV), or one of three Globally Harmonized System (GHS) categories (1, 2A, or 2B) with substances that do not fall into one of the three GHS categories considered not classified by GHS [5]. A summary of the EPA and GHS categories can be found in Choksi et al. [13]. The rabbit test was never validated for its relevance to humans. There are numerous differences between rabbit and human eyes; for example, unlike humans, rabbits have a nictitating membrane, which can serve either to remove irritants from the surface of the eye or trap them there [14,15]. In addition, the pH of a rabbit eye aqueous humour is more alkaline (8.2) than that of a human eye (7.1–7.2) which may account for the greater susceptibility of the rabbit iris to chemical irritation relative to humans [16]. Rabbits also are not as efficient in tear production as humans, therefore, the severity of effects may differ between species [17]. Furthermore, unlike GHS, the EPA system conservatively uses the most severe single animal response for categorization, making comparisons between rabbit and *in vitro/ex vivo* results nearly impossible. *Reproducibility and repeatability:* Reproducibility issues with the test as well as the subjective nature of assessments limit its reliability [1,18–21]. For example, an analysis of almost 500 chemicals for which multiple rabbit eye test studies were available indicates that chemicals identified as GHS category 2A or 2B in one test are more likely to not be classified by GHS than be GHS category 2 in a subsequent test [21]. Studies have also demonstrated the impact on classification of variability in individual endpoints scores within a single test. Although not a robust prospective assessment of variability (as has been done for many of the alternative methods summarized herein), resampling analysis showed that chemicals classified as GHS category 2 have a 12% probability of not being classified by GHS, and GHS category 1 have an 11% chance of being identified at GHS category 2. Additionally, repeat tests on the same chemical resulted in the same GHS classification for only 6/16 chemicals that were classified as Cat 1 in at least one test [21,22].	*Test system*: The test is conducted in live rabbits and specific to mode of actions for rabbit eyes [1]. *Mode of action*: The test evaluates apical outcomes in the rabbit eye, including corneal opacity, conjunctival changes, and iritis (applicable to Figure 2(c–g)). Since only apical outcomes are determined, the test provides limited mechanistic information, and due to interspecies differences in ocular anatomy and physiology, modes of action elucidated by the rabbit test may not represent the mechanisms that occur in human cells or tissue. *Depth of injury concept:* The tissue structure, thickness, and biochemistry of human and rabbit cornea differ and may result in differences in irritation responses between the two species [23–26]. Aside from interspecies differences, the intact rabbit eye with surrounding conjunctival tissues provides an analogous "full thickness" model with most of the essential corneal and conjunctival tissue layers found in the intact human eye. *Exposure time and washing:* According to EPA TG OPPTS 870.2400 and OECD TG 405, one eye of one or more rabbits is exposed to a single dose for 24 h or more, and eyes are examined at 1, 24, 48, and 72 h, and up to 21 days, after application. The eye may be washed after 24 h (1 h for solids, if applying OECD TG 405) or earlier for immediate corrosive effects. The exposure time is longer than an anticipated human exposure, which is thought to contribute to over-prediction of eye effects. If no irritation has occurred after 72 h, the study may be terminated [4,5]. *Reversibility:* According to EPA TG OPPTS 870.2400 and OECD TG 405, if the animals suffer from minor lesions, qualitative measurement of reversibility of effects is assessed at various time points up until 21 days; however, if reversibility is observed prior to this, the study may be terminated [5].
Reconstructed 3D human tissue assays		
OECD TG 492: Reconstructed Human Cornea-like Epithelium (RhCE) test [27]	The test quantitatively measures a substance's ability to induce cytotoxicity in a 3D reconstructed human cornea-like epithelial tissue. Although cell damage may occur via multiple modes of action, cytotoxicity plays a primary role in determining overall ocular damage [27]. When OECD TG 492 was first published in 2015, the only model included was EpiOcular™, which is 5–8 cell layers of normal human epidermal	*Test system*: The test uses relevant cells from the species of interest in an *in vitro* 3D reconstructed human corneal epithelium model cultured at air interface [1]. *Mode of action*: The RhCE tissues model essential events in the corneal epithelium (i.e. breach of barrier function and epithelial cell death in Figure 2(c–e)), by measuring changes in cellular metabolic rate after a fixed exposure (e.g. MTT

(*continued*)

	keratinocytes. SkinEthic™ HCE (made from transformed human corneal epithelial cells) [28,29], LabCyte [30], and MCTT HCE™ [31] (the latter two made from normal human corneal epithelial cells) followed. These tissues are histologically, morphologically, biochemically, and physiologically similar to *in vivo* human corneal epithelial cells [1]. *Classification:* Chemicals which do not reduce tissue viability below the test method-specific threshold post-exposure can be identified as not classified by GHS (using a bottom-up approach). This method has not been formally evaluated for its ability to classify substances into EPA categorizations. *Reproducibility and repeatability*: Validation studies demonstrated the RhCE methods to be highly reproducible. For example, within and between laboratory reproducibility for EpiOcular™ was 95% and 93% [27]. *Proficiency testing*: Prior to routine use of this method, laboratories must test fifteen set substances to prove technical proficiency. The false negative rates of these methods are within the *in vivo* rabbit within-test variability rate of 12% [21]. To fulfil OECD guidelines, variability of tissue replicates for positive/negative controls and for test substances must fall within acceptable bounds i.e. the difference of viability between two tissue replicates should be less than 20%. To identify both substances causing serious eye damage and substances not requiring classification, additional protocols were developed (see below).	assay) where discrimination between non-irritants and irritants is relevant. *Depth of injury concept:* These tissues model the stratified corneal epithelium and, therefore, are adept at identifying non-irritants, and discriminating between irritants and non-irritants. *Exposure time and washing:* Prior to exposure, the tissue is moistened to mimic conditions of the human eye. For OECD TG 492, exposure times vary for different RhCE types. For liquids, the time range is between 1 and 30 min. For solids, the time range is between 3 and 24 h. After exposure, the RhCE is thoroughly rinsed [27]. *Reversibility*: Reversibility of lesions is not assessed in this method.
Time-to-Toxicity (ET50) protocols using RhCE models [32,33]	The test quantitatively measures a substance's ability to induce cytotoxicity in a 3D reconstructed human cornea-like epithelial tissue which is histologically, morphologically, biochemically, and physiologically similar to *in vivo* human corneal epithelial cells. EpiOcular™ has been used in this protocol [32,33] as well as SkinEthic [34]. Although cell damage may occur via multiple modes of action, cytotoxicity plays a primary role in determining overall ocular damage. *Classification:* The test was optimized to identify EPA category I (using a top-down approach), III and IV (using a bottom-up approach) cleaning products with antimicrobial claims by estimating the exposure time required to reduce tissue viability by 50% (ET50). EPA category I chemicals have an ET50 $<$ 4 min, category III chemicals have an ET50 of $\geq$4 min and $<$70 min, and category IV chemicals have an ET50 $\geq$ 70 min [32]. Additionally, method developers suggest that the test can be used to predict GHS categories 1 and 2 and to identify substances not classified by GHS (e.g. ET50 $<$ 8 min, $\geq$8 but $<$80 min, and $>$80 min, respectively, for surfactants). Note a slightly different terminology used by EPA versus GHS; EPA category I is defined as corrosives while GHS category 1 is defined as substances causing serious eye damage. *Reproducibility and repeatability*: The reproducibility of predictions between two independent runs of 78 and 79 chemicals was 82.3% and 91% for neat and diluted protocols, respectively. Reproducibility may be slightly lower for substances with very low ET50 values due to limitations of washing substances off the tissue. In some cases, this may be solved by diluting the substance [33]. Information on repeatability is not currently available.	*Test system*: The test uses relevant cells from the species of interest in an *in vitro* 3D reconstructed human corneal epithelium model cultured at air interface [33]. *Mode of action*: The RhCE tissues model essential events in the corneal epithelium (i.e. breach of barrier function and epithelial cell death in Figure 2(c–e)) by measuring exposure time-related changes in cellular metabolic rate (e.g. MTT assay) and can discriminate between non, minimal, and moderate irritants (and provide some information to identify severe irritants/corrosives). *Depth of injury concept:* These tissues model the stratified corneal epithelium and, therefore, are adept at identifying non-irritants, and discriminating between irritants and non-irritants. The Time-to-Toxicity protocol allows for a continuum of exposure-time related cytotoxic responses that correlate with the cell death events associated with stromal injuries *in vivo*. *Exposure time and washing:* To predict EPA categories, standard exposure times of 2, 15, 45, and 90 min are used. After exposure, the RhCE is thoroughly rinsed [32]. To predict GHS classifications, liquids are exposed neat for 3 min and at 20% dilution for 16 and 256 min. For solids in 1:1 dispersion, exposure times of 64 and 256 min are used [33]. *Reversibility*: Reversibility of lesions is not assessed in this method.
EYEIRR-IS® [35–37]	The test quantitatively measures a substance's ability to cause corneal injury as well as assessing	*Test system*: The assay employs a 3D reconstructed human corneal epithelial model, such as

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	reversibility. The assay monitors the expression of biomarkers that play roles in wound healing, interleukin activation, corneal neovascularization, and tear film [36]. The genes analyzed and overexpression thresholds as well as the algorithm used can be found in Cottrez et al [37]. *Classification:* Although not formally adopted by the OECD, decision criteria have been proposed by the method developers to distinguish between GHS categories 1 and 2 (using a top-down approach) and not classified. Users should consult with relevant authorities to determine the extent to which they might be considered acceptable for that purpose. This method has not been evaluated for its ability to classify substances into EPA categorizations. *Reproducibility and repeatability:* This method has not been through a formal multi-laboratory validation study. Information on reproducibility and repeatability are not currently available.	SkinEthic™ human corneal epithelium cultured *in vitro* at the air interface, using relevant cells from the species of interest [1]. *Mode of action*: The test measures expression of biomarkers, which have been shown to overexpress in the presence of irritants. This assay is relevant to the changes in biomarker expression and cell death in epithelial cells in Figure 2(c–e) (injury involving the conjunctival or corneal epithelium) using relevant cells from the species of interest. *Depth of injury concept:* This system uses 3D reconstructed human tissues that model the stratified corneal epithelium and, therefore, are adept at identifying non-irritants, and discriminating between irritants and non-irritants. *Exposure time and washing:* The tissue models are exposed to liquid substances for 10 min and to solid substances for 30 min. Phosphate-buffed saline is applied to the edge of the insert (not directly to the tissue) to remove the substance. *Reversibility*: Potency and reversibility are assessed by measuring expression of relevant genes in the cells [36].
OECD TG 494: Vitrigel-Eye Irritancy test (EIT) [38]	The test quantitatively measures a substance's ability to induce injury to the barrier function of human corneal epithelial (hCE) cells, assessed by analyzing time-dependent changes in transepithelial electrical resistance (TEER) values. *Classification:* The method can be used to identify substances not classified by GHS (using a bottom-up approach). This method has not been evaluated for its ability to classify substances into EPA categorizations. *Reproducibility and repeatability*: The test has been scientifically validated. The reproducibility between three laboratories was 92%. Within-laboratory reproducibility was 80–100% at the three laboratories that conducted the validation study. *Proficiency testing*: Prior to routine use of this method, laboratories must test ten set substances to prove technical proficiency [38].	*Test system*: The test uses human corneal epithelial cells cultured *in vitro* to form a uniform squamous epithelium in a collagen vitrigel membrane chamber at air interface, thus using relevant cells from the species of interest [1]. *Mode of action*: The test assesses injury to the epithelial barrier function by measuring loss of TEER (i.e. injury to tight junctions and reduction in epithelial barrier function in Figure 2(c,d)) [39]. *Depth of injury concept:* This tissue models the squamous epithelium and does not mechanistically model deeper stromal or endothelial damage, and therefore, is limited to discriminating between non-irritants and irritants. *Exposure time and washing:* Cells are exposed to the substance for 3 min, with TEER measurements taken every ten seconds during exposure [38]. The short exposure time is designed to reflect the fact that substances are usually flushed from the human eye within minutes after exposure. *Reversibility*: Reversibility of lesions is not assessed in this method.
Organotypic *ex vivo* assays		
OECD TG 437: Bovine Corneal Opacity and Permeability (BCOP) test method [40] with the optional addition of histopathology analysis	The test quantitatively measures a substance's ability to induce opacity and increased permeability in the cornea of cow eyes obtained as by-products from slaughterhouses. The opacity and permeability assessments are combined to derive an In Vitro Irritancy Score (IVIS), which is used to classify the irritancy level of the test chemical. *Classification:* The test was optimized to identify cleaning products with antimicrobial claims falling under EPA categories I, II (using a top-down approach), and III (using a bottom-up approach) (i.e. IVIS of $\geq$75, $>$25 and $<$75, or $<$25, respectively) [32]. Additionally, the test can be used to identify chemicals under GHS category 1 or those not classified by GHS [40]. *Reproducibility and repeatability*: The test has been scientifically validated. Concordance of results was seen for 67–100% of severely irritating (corrosive)	*Test system*: The test uses *ex vivo* bovine corneas that are mounted within corneal holders and maintained in culture medium. The assay addresses corneal effects (the main contributor to *in vivo* classification) by objectively measuring induction of corneal opacity and breakdown of the corneal barrier function – events that are readily associated with numerous modes of chemical action resulting in cell necrosis and tissue erosion (cell membrane lysis, saponification, cellular and collagen/protein coagulation, precipitation). Further information on chemical interactions with macromolecules that pre-empt delayed cell death may be available on addition of histopathology analysis [1]. *Mode of action*: This assay is applicable to Figure 2(c–e) (injury involving the conjunctival or corneal epithelium), Figure 2(f) (damage

(*continued*)

	substances tested across three laboratories [41–43]. The per cent coefficient of variance (CV) was 1.1–18.1% for severe eye irritants (corrosives) [44]. *Proficiency testing*: Prior to routine use of this method, laboratories must test thirteen set substances to prove technical proficiency.	involving the corneal stroma), and, with addition of histopathology, Figure 2(g) (damage involving the endothelium). *Depth of injury concept:* Bovine corneas differ structurally from human corneas, for example, they are thicker and do not contain a Bowman's layer; however, their similar morphology makes them a useful full-thickness model to predict human effects. Histopathology can be conducted for additional information, including details on the DOI, and delayed effects not captured by the initial opacity and loss of barrier function endpoints alone [40,45,46]. Evaluating histological changes in the endothelial and stromal layers can further support discrimination between moderate and severe irritants (corrosives). *Exposure time and washing:* The corneas are washed with pre-warmed media prior to exposure. Typically, corneas are exposed to liquids, semi-solids, creams and waxes for 10 min, and to non-surfactant solids for 4 h; however, exposure times may vary for particular substances. After exposure, the corneas are washed at least three times. *Reversibility*: Histopathology can be conducted to determine the depth and degree of injury to support predictions of reversibility [40,45,46].
OECD TG 438: Isolated Chicken Eye (ICE) test [47]	The test quantitatively measures a substance's ability to induce corneal swelling, and subjectively measures its ability to induce corneal opacity, and injury to the epithelium (measure of fluorescein retention) of chicken eyes obtained as by-products from slaughterhouses. These individual assessments are assigned qualitative categorizations that, when combined, provide a classification. *Classification:* The method can be used for the identification of substances (solids, liquids, emulsions and gels) causing serious eye damage (EPA Category 1, GHS category 1; using a top-down approach) and substances causing minimal effects (those not classified by GHS; using a bottom-up approach) [32,47]. *Reproducibility and repeatability*: The test has been scientifically validated. Seventy percent of EPA category I substances showed concordance across all four laboratories involved in validation [48]. Although within-test variability is low for corneal thickness measurements (range of coefficient of variance values from 0.9% to 6.1%), variability within other measurements was higher. *Proficiency testing*: Prior to routine use of this method, laboratories must test thirteen set substances to prove technical proficiency [47].	*Test system*: The test uses *ex vivo* chicken *eyes* that are mounted on specialized whole globe eye holders. The test addresses corneal effects (the main contributor to *in vivo* classification) in the corneas by objectively measuring induction of corneal opacity and breakdown of the corneal barrier function, events that are readily associated with numerous modes of chemical action resulting in cell necrosis and tissue erosion (cell membrane lysis, saponification, cellular and collagen/protein coagulation, precipitation) [49]. Further information on chemical interactions with macromolecules may be available on addition of histopathology analysis [1]. *Mode of action*: This assay is applicable to Figure 2(c–e) (injury involving the conjunctival or corneal epithelium) and Figure 2(f) (damage involving the corneal stroma), and, with addition of histopathology, provides some insights into Figure 2(g) (damage involving the endothelium). *Depth of injury concept*: Chicken corneas differ structurally from human corneas; however, their similar morphology makes them a useful full-thickness model to predict human effects. Histopathology can be conducted for additional information about the DOI [45,47,50]. Evaluating histological changes in the endothelial and stromal layers can further support discrimination between moderate and severe irritants (corrosives). *Exposure time and washing:* Corneas are exposed to liquids or solids for 10 s, then washed until the substance has been removed. *Reversibility*: Per TG 438, histopathology can be conducted to determine the depth and degree of injury to support predictions of reversibility [45,47,50].
Isolated Rabbit Eye (IRE) test [51,52]	The test quantitatively measures a substance's ability to induce corneal swelling, and subjectively measures its ability to induce corneal opacity, and injury to the epithelium (measure of fluorescein	*Test system*: The test uses *ex vivo* rabbit eyes that are mounted on specialized whole globe eye or corneal holders. The test addresses corneal effects (the main contributor to *in vivo*

(*continued*)

	retention) of rabbit eyes, which have historically been obtained either as by-products from slaughterhouses or from laboratories. These individual assessments are assigned qualitative categorizations that, when combined, provide an eye irritation potential. *Classification:* The method can be used to identify substances causing serious eye damage (GHS category 1; using a top-down approach) [1]. This method has not been evaluated for its ability to classify substances into EPA categorizations. *Reproducibility and repeatability*: The qualitative nature of the assay limits assessment of the reproducibility and repeatability of the IRE test method. While not an OECD TG, this method was assessed in a 37-laboratory international validation effort [33].	classification) as well as protein coagulation (precipitation/denaturation of macromolecules) in the cornea [53]. The IRE can produce quantitative measurements and better control exposure time and volume *ex vivo*, each of which reduces possible variability as compared to the live rabbit test. *Mode of action*: This assay is applicable to Figure 2(c–e) (injury involving the conjunctival or corneal epithelium) and Figure 2(f) (damage involving the corneal stroma), and, with addition of histopathology, Figure 2(g) (damage involving the endothelium). *Depth of injury concept:* Rabbit corneas differ structurally from human corneas; however, their similar morphology makes them a useful full-thickness model to predict human effects. Histopathology can be conducted for additional information about the DOI. Evaluating histological changes in the endothelial and stromal layers can further support discrimination between moderate and severe irritants (corrosives). *Exposure time and washing:* Pre-warmed media is added to rabbit eyes prior to exposure to the substance. The eyes are exposed to the test substance for 10 s to identify a severe irritant (corrosive) and for 1 min (or longer) for less severely irritating substances [54,55]. They are washed until the test substance is removed, and assessments are made at several further time points. *Reversibility*: Reversibility of lesions is not typically assessed in this method, but histopathology could be conducted to determine the depth and degree of injury to support predictions of reversibility.
Porcine cornea opacity/reversibility assay (PorCORA) [56,57]	This test quantitatively measures a substance's ability to cause corneal injury and assesses reversibility. Porcine corneal cells are extracted from pig eyes, which are obtained as by-products from slaughterhouses. The cells are sustained for 21 days, using an air-interface, and injury to the epithelial barrier function is detected using a sodium fluorescein stain. *Classification:* Although not formally adopted by the OECD, decision criteria have been proposed by the method developers to distinguish between EPA categories I and II, or GHS categories 1 and 2 (using a top-down approach) [56,57]. Users should consult with relevant authorities to determine the extent to which they might be considered acceptable for that purpose. *Reproducibility and repeatability:* This approach has not been through a formal multi-laboratory validation study. Information on reproducibility and repeatability is not currently available.	*Test system*: The test uses *ex vivo* porcine corneas which are cultured at air-interface on a soft agar:culture medium matrix to maintain viability of the epithelium. *Mode of action*: The test assesses injury to and subsequent recovery of the epithelial barrier in *ex vivo* porcine corneal tissues. This assay is applicable to Figure 2(c–e) (injury involving the conjunctival or corneal epithelium). *Depth of injury concept*: While there are structural differences between human and pig eyes, the porcine cornea is roughly the same thickness as a human cornea, and is generally regarded as one of the most human-like architecturally among common test species. Although the test uses full-thickness porcine corneas complete with epithelial, stromal, and endothelial layers, the test only demonstrates recovery of the corneal epithelium and does not address recovery of the stromal elements. However, by evaluating histological changes in the stromal and endothelial layers within a few hours after chemical exposure, an assessment of the depth and degree of initial injury may further support discrimination between moderate and severe irritants (corrosives). Whereas information to support reversibility predictions could be gleaned from the BCOP, ICE, and IRE assays by conducting histopathology on the corneas after the standard assay is completed, histopathology would generally need to be conducted in porcine corneas within a few hours after chemical exposure, which could be conducted in parallel to the PorCORA.

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		Exposure time and washing: The cells are exposed to the substance for 5 min, rinsed with phosphate-buffered saline, and then incubated for a further 21 days so reversibility can be assessed [56,57]. *Reversibility*: While this method can be used to evaluate reversible effects, those reversible events are limited to demonstration within the epithelium, which mechanistically provides only limited relevance to the issues of reversibility in both the rabbit and human corneas. Specifically, recoverability of the epithelial injury *in vitro* can occur even when the stromal elements do not show recovery; the latter of which are frequently critical to discriminating between moderate and severe irritants (corrosives).
Ex Vivo Eye Irritation (EVEIT) test [58]	This test measures a substance's ability to cause corneal injury and assesses reversibility. Rabbit corneas are extracted from rabbit eyes, which are obtained as by-products from slaughterhouses. The cells are sustained for 72 h, and injury to the epithelial barrier function is detected using a sodium fluorescein stain. In addition, optical coherence tomography (OCT) is conducted at multiple times during an experiment to non-invasively assess epithelium and stromal injury and recovery. *Classification*: Although not formally adopted by the OECD, decision criteria have been proposed by the method developers to distinguish between GHS categories 1 and 2 or those not classified by GHS. Users should consult with relevant authorities to determine the extent to which they might be considered acceptable for that purpose. This method has not been evaluated for its ability to classify substances into EPA categorizations. *Reproducibility and repeatability*: This approach has not been through a formal multi-laboratory validation study. Information on reproducibility is not currently available, however the within-laboratory repeatability for 37 chemicals assessed by the method developers was 97.3% [58].	*Test system*: The test uses *ex vivo* rabbit corneas cultured at the air-interface on a soft agar:culture medium matrix to maintain viability of the epithelium. *Mode of action*: The test assesses injury to and subsequent recovery of the epithelial barrier in *ex vivo* rabbit corneal tissues. This assay is applicable to Figure 2(c–e) (injury involving the conjunctival or corneal epithelium), Figure 2(f) (damage involving the corneal stroma), and Figure 2(g) (damage involving the endothelium). *Depth of injury concept*: Rabbit corneas differ structurally from human corneas; however, their similar morphology makes them a useful full-thickness model to predict human effects. Histopathology can be conducted for additional information about the DOI. Evaluating histological changes in the endothelial and stromal layers can further support discrimination between moderate and severe irritants (corrosives). *Exposure time and washing:* The cells are exposed to solid substances for an hour and liquid substances for 30 s. *Reversibility*: This method was designed to evaluate reversibility based upon evaluating the epithelium, stroma, and endothelium [58].
Cytotoxicity and cell function based *in vitro* assays		
OECD TG 460: Fluorescein Leakage (FL) test [59]	The test quantitatively measures a substance's ability to induce increased permeability of sodium fluorescein through a confluent monolayer of Madin-Darby Canine Kidney (MDCK) CB997 tubular epithelial cells. *Classification:* The method can be used to quantitatively identify substances causing serious eye damage (EPA category I or GHS category 1; using a top-down approach). The test has been scientifically validated. *Reproducibility and repeatability*: According to the validation study, 89% of chemicals were identically classified by the two laboratories. Within-laboratory variability ranged from CV (coefficient of variance) = 28–36% [60]. *Proficiency testing*: Prior to routine use of this method, laboratories must test eight set substances to prove technical proficiency [59].	*Test system*: Canine kidney cells are cultured *in vitro* as a confluent monolayer on permeable tissue culture inserts generally maintained in culture medium. *Mode of action*: The test assesses injury to tight and desmosomal junctions within canine kidney cells. Damage to these junctions can lead to trans-epithelial permeability and subsequent ocular irritation [1]. This assay is applicable to Figure 2(c) (injury limited to the superficial conjunctival or corneal epithelium). Although this model is limited mechanistically to the loss of barrier function of the squamous epithelium, and thus is not mechanistically linked to the events that define moderate and severe irritants (corrosives), the range of responses allow this method to be correlatively linked in the prediction of severe irritants (corrosives). *Depth of injury concept:* This test system models the upper squamous epithelium, and is

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		therefore limited to modelling loss or erosion of epithelial barrier function, and does not mechanistically model deeper stromal or endothelial damage. *Exposure time and washing:* Washing of the monolayer of cells with pre-warmed salt solution is conducted prior to exposure. Cells are exposed to the substance for 1 min, which reflects the short exposure time and clearance rate anticipated in a human exposure. The substance is carefully removed by aspiration and the cells are washed twice with pre-warmed salt solution before immediate fluorescein measurements are taken. *Reversibility*: Reversibility of lesions is not assessed in this method.
OECD TG 491: Short Time Exposure (STE) *in vitro* test [61,62]	The test quantitatively measures a substance's ability to induce cytotoxicity in a confluent monolayer of Statens Seruminstitut Rabbit Cornea (SIRC) cells. Although cell damage may occur via multiple modes of action, cytotoxicity plays a primary role in determining overall ocular damage. *Classification:* According to the 2013 NICEATM STE summary review document, the method can be used to quantitatively identify substances under EPA category I (using a top-down approach) or IV (using a bottom-up approach) [63]. Additionally, the test can be used to identify substances causing serious eye damage (GHS category 1) and or those not classified by GHS [61]. *Reproducibility and repeatability:* The test has been scientifically validated. According the ICCVAM evaluation, for interlaboratory agreement, the laboratories recorded 100% agreement for 83–100% and 87–100% of the substances for GHS and EPA classification, respectively. Within-laboratory variability ranged from CV (coefficient of variance) = 0.3–23.5% [63]. *Proficiency testing*: Prior to routine use of the STE method, laboratories must test eleven set substances to prove technical proficiency.	*Test system*: Rabbit cornea (SIRC) cells (a rabbit corneal fibroblast cell line used as a model of the squamous ocular epithelium) are cultured *in vitro* as a 2D monolayer in multiwall tissue culture plates immersed in culture medium. *Mode of action*: The test assesses changes in cellular metabolic rate (e.g. MTT assay) in the SIRC cells that can lead to epithelial injury and eye irritation [1]. This assay is relevant to the epithelial cell death key event in Figure 2(c) (damage limited to the superficial conjunctival or corneal epithelium). Although this model is limited mechanistically to epithelial cell death, and thus is not mechanistically linked to the events that define moderate and severe irritants (corrosives), the range of responses allow this method to be correlatively linked in the prediction of severe irritants (corrosives). *Depth of injury concept:* This test system models the upper squamous epithelium, and therefore is limited to modelling loss or erosion of epithelial barrier function, and does not mechanistically model deeper stromal or endothelial damage. *Exposure time and washing:* Cells are exposed to the substance for 5 min, prior to washing with phosphate-buffered saline [61]. The short exposure time is designed to reflect the fact that substances are usually flushed from the human eye within minutes after exposure. *Reversibility*: Reversibility of lesions is not assessed in this method.
Neutral Red Release (NRR) test [64,65]	The test quantitatively measures a substance's ability to induce damage to cell membranes in a monolayer of normal human epidermal keratinocytes (NHEK). Initially, cells are incubated in a dye (neutral red), which accumulates solely within lysosomes. If the cells are exposed to irritants, the cell membranes are disturbed. A quantitative assessment of the release of dye allows for categorization of ocular irritation [65]. The test is high-throughput and provides information on the concentration–response relationship [9]. *Classification:* Although not formally adopted by the OECD, decision criteria have been proposed by the method developers to identify substances causing minimal effects (those not classified by GHS; using a bottom-up approach), and may also be useful for identifying agrochemical formulations causing serious eye damage (GHS category 1; using a top-down approach) [9]. Users should consult with relevant authorities to determine the extent to which they might be considered acceptable for	*Test system*: Human epidermal keratinocytes are cultured *in vitro* as a 2D monolayer in multiwall tissue culture plates immersed in culture medium as a model of the corneal squamous epithelium using cells from the species of interest [1]. *Mode of action*: The test assesses cell membrane lysis in a monolayer of undifferentiated epithelial cells. This assay is relevant to the epithelial cell death key event in Figure 2(c) (injury limited to the superficial conjunctival or corneal epithelium) using cells from the species of interest. *Depth of injury concept:* This test system models the upper squamous epithelium, and therefore is limited to modelling loss or erosion of epithelial barrier function and does not mechanistically model deeper stromal or endothelial damage. *Exposure time and washing:* Prior to exposure, the

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	that purpose. This method has not been evaluated for its ability to classify substances into EPA categorizations. This approach went through a retrospective validation study and was peer reviewed by ESAC. Although ESAC did not recommend the method for routine use, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) concluded that the assay could provide "extremely valuable information when it was used for particular purposes." For example, to identify substances that are "potentially capable of causing adverse reactions on coming into brief contact with the eye or the skin at relatively high concentrations, such as might occur in an adventitious splash into the eye or onto the skin, followed by a quick rinse" [66]. *Reproducibility and repeatability:* Information on reproducibility and repeatability is not currently available.	cells are incubated with neutral red dye for 3 h, then washed before the substance is added. Cells are exposed to the chemical for 1 min, then washed up to three times to remove neutral red dye ejected from lysed cells [9]. *Reversibility*: Reversibility of lesions is not assessed in this method.
Cytosensor Microphysiometer (CM) test [67]	The test quantitatively measures the concentration of test material that causes a 50% decrease in the acidification rate (MRD_{50}) in a sub-confluent monolayer of mouse L929 fibroblasts, using a pH metre to detect changes in acidity. *Classification:* The test has been scientifically validated, and can be used to quantitatively identify cleaning products with antimicrobial claims under EPA categories I, III, and IV (MRD_{50} scores of <2 mg/mL, ≥ 2 but <80 mg/mL, and ≥ 80 mg/mL, respectively) [32]. Additionally, the test can be used to identify GHS category 1 substances (using a top-down approach) or those not classified by GHS (using a bottom-up approach) [67]. *Reproducibility and repeatability*: Inter-laboratory reproducibility for bottom-up assessments were 100% for GHS classification and 94.44% for EPA, and, for top-down assessments, were 87.62% for GHS with no values provided for EPA classification. Intra-laboratory repeatability was assessed based on calculated CVs for MRD_{50} scores from two different studies. Mean CVs tended to be higher for surfactant substances than non-surfactant substances, ranging from 10% to 24% [48]. Although this assay demonstrated promise and was incorporated into the alternate testing framework for classification of eye irritation potential of EPA pesticide products, this test requires specialized equipment that currently is not readily available.	*Test system*: Mouse L929 fibroblasts are cultured *in vitro* as a confluent monolayer on permeable tissue culture inserts immersed in culture medium. *Mode of action*: This assay is relevant to the epithelial cell death key event in Figure 2(c) (damage limited to the superficial conjunctival or corneal epithelium) by assessing changes in cellular metabolic rate (e.g. by changes in release of acidic metabolites). Although this model is limited mechanistically to epithelial cell death, and thus is not mechanistically linked to the events that define moderate and severe irritants (corrosives), the range of responses allow this method to be correlatively linked in the prediction of severe irritants (corrosives). *Depth of injury concept:* Since this test system is limited to modelling the upper squamous epithelium, it does not mechanistically model deeper stromal or endothelial damage. *Exposure time and washing:* Increasing concentrations of the substance are introduced, via flow-through, to the cells over 13 min 30 s, then the cells are washed [67]. *Reversibility*: Reversibility of lesions is not assessed in this method.
Macromolecular matrix assays		
OECD TG 496: *In vitro* Macromolecular Test Method Ocular Irritection® [68]	The test quantitatively assesses a test substance's potential to cause serious eye damage by measuring the extent of denaturation (measure of turbidity) within a molecular matrix exposed to the substance (via optical density readings). The topical application of irritant substances induces changes of the matrix (turbidity) which are measured by optical density. An increase in optical density is used to predict the ocular hazard effects of chemicals based on the premise that corneal opacity observed *in vivo* may result from the disruptive effects ocular irritants can have on the highly organized structure of the cornea through interaction with some of its components (e.g. proteins and carbohydrates). *Classification:* The test can be used to identify GHS category 1 substances (using a top-down approach), and those not classified by GHS (using a bottom-up approach) [1,68]. This method has not been evaluated for its ability to classify substances	*Test system*: The test is an *in chemico* macromolecular matrix model composed of lipids, proteins, glycoproteins, and low molecular weight substances that model the cellular components of the cornea. Although the test system is not a live biological system, the macromolecular matrix provides an analogous model of the organized biochemistry in the corneal epithelium and stroma. *Mode of action*: The test addresses the denaturation of the macromolecular matrix as a model of protein and lipid denaturation in corneal tissues. This assay is applicable to the key events of protein and lipid denaturation in Figure 2(c–e) (injury involving the conjunctival or corneal epithelium) and Figure 2(f), (damage involving the corneal stroma). *Depth of injury concept:* Although the model uses a 3D matrix, there is no stratification or organized

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	into EPA categories. *Reproducibility and repeatability*: The *in vitro* macromolecular test method Irritection® has been scientifically validated [69], and underwent peer-review by the EURL ECVAM Scientific Advisory Committee [70]. The within-laboratory repeatability was 88.2%, 84.0%, and 80.4% (when a cut-off of 12.5 was used) or 90.2%, 86.0%, and 84.3% (when a cut-off of 30 was used). The between-laboratory reproducibility was 84.0% (when a cut-off of 12.5 was used) and 86% (when a cut-off of 30 was used). *Proficiency testing*: Prior to routine use of this method, laboratories must test 12 set substances to prove technical proficiency [68].	layering relevant to the corneal or conjunctival architectures and hence it does not model DOI. *Exposure time and washing:* A test chemical is applied to the macromolecular matrix, which is then incubated for 24 h at 25 °C [68,71]. *Reversibility*: Reversibility of lesions is not assessed in this method.
OptiSafe test [10,72]	This test quantitatively assesses a test substance's potential to cause eye irritation by measuring damage to macromolecules. *Classification:* Although not formally adopted by the OECD, decision criteria have been proposed by the method developers to identify substances causing minimal effects (EPA category IV or those not classified by GHS; using a bottom-up approach), and may be useful for identifying agrochemical formulations causing serious eye damage (EPA category I or GHS category 1; using a top-down approach) [72]. Users should consult with relevant authorities to determine the extent to which they might be considered acceptable for that purpose. *Reproducibility and repeatability*: This approach has not yet been through an official validation phase; however, a validation study showed inter-laboratory reproducibility of 91% for both EPA and GHS classifications [72]. Intra-laboratory reproducibility was greater than 90% for identification of ocular irritants [73].	*Test system*: The test is an *in chemico* macromolecular matrix model composed of lipids, proteins, glycoproteins, and low molecular weight substances that model the cellular components of the cornea. Although the test system is not a live biological system, the macromolecular matrix provides an analogous model of the organized biochemistry in the corneal epithelium and stroma. *Mode of action*: The test addresses the denaturation of the macromolecular matrix as a model of protein and lipid denaturation in corneal tissues. This assay is applicable to the key events of protein and lipid denaturation in Figure 2(c–e) (injury involving the conjunctival or corneal epithelium), and Figure 2(f), (damage involving the corneal stroma). *Depth of injury concept:* Although the model uses a 3 D matrix, there is no stratification or organized layering relevant to the corneal or conjunctival architectures and hence it does not model the DOI. *Exposure time and washing:* The active agent is exposed to the substance for 10–15 min or 18 h, depending on results from a pre-test [72]. *Reversibility*: Reversibility of lesions is not assessed in this method.

anterior limiting lamina. The anterior limiting lamina in chickens is most similar to the Bowman's layer in humans [83]. All of the corneal layers have their own function and play a different role in the progression and recovery of corneal damage.

The conjunctival tissues are composed of an epithelium overlaying the vascular *lamina propria*. Conjunctival tissues are continuous from the anterior corneal–scleral limbus of the eye, where the conjunctival tissues overlaying the eye globe are referred to as the bulbar conjunctiva, to the outer edges of the eyelids, where the tissues are referred to as the palpebral conjunctiva. The palpebral conjunctiva overlays various glands including tarsal and Meibomian glands, and is populated with goblet cells [84]. Humans have a smaller conjunctival cul-de-sac than rabbits and, therefore, have a notably lower likelihood of retention of a substance following exposure. Whereas the rabbit cul-de-sac is estimated to hold between 30 and 50 μL [85], the human cul-de-sac is estimated to normally hold 7–10 μL of tears and potentially hold up to 25 μL volume in total [86].

Many of the *in vitro* and *ex vivo* test methods model the cornea, or specific layers of the cornea. Since the superficial epithelial tissues in the cornea and conjunctiva have similar morphology, *ex vivo* corneal models or *in vitro* corneal epithelial tissue models can also be reasonable surrogates for evaluating irritation to the epithelium of the conjunctiva. To demonstrate the relevance, the loss of the squamous and upper wing cell layers in an *ex vivo* bovine corneal model by surfactant-based formulations coincided with mild to moderate injury to the conjunctiva of the rabbit *in vivo* [87]. Essentially the same mechanisms that cause surfactant erosion and penetration of the stratified epithelium of the cornea also affect the stratified epithelium of the conjunctivae.

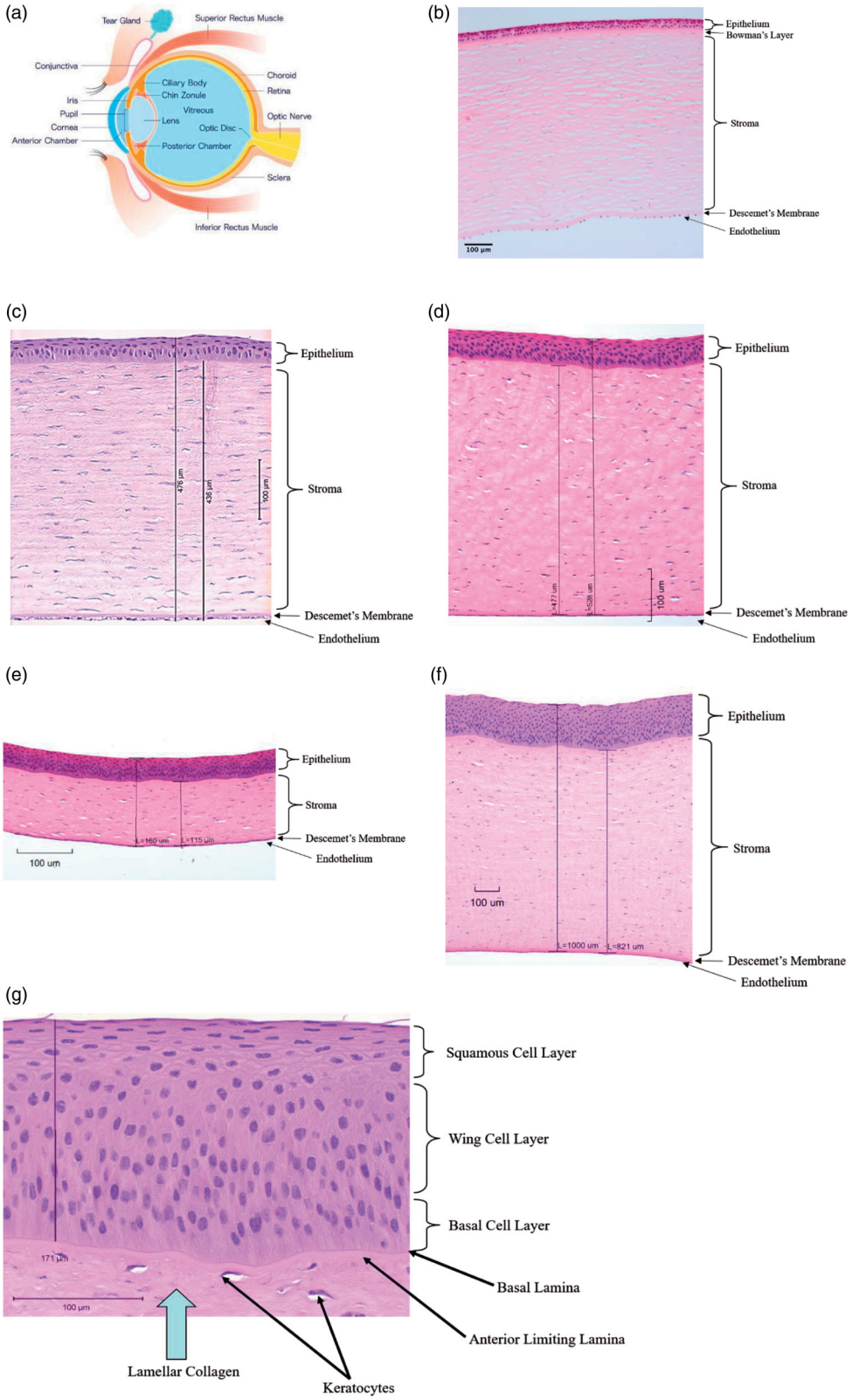

Figure 1. Comparison of human, rabbit, porcine, chicken and bovine corneas. (a) Schematic depiction of the human eye (image purchased from iStock). (b) Healthy human cornea section (image courtesy of Hans E. Grossniklaus, MD), (c) rabbit cornea section (obtained after whole globe immersion in fixative), (d) dissected pig cornea section, and (e) dissected chicken cornea section. (f) Full thickness bovine cornea from the bovine corneal opacity and permeability (BCOP) assay (sterile, deionized water, 10-min exposure, 2-h post exposure). (g) Epithelium and upper stroma of a bovine cornea from the BCOP assay showing the three epithelial tissue layers, the collagen-rich acellular anterior limiting lamina between the basal lamina, and the organized stromal collagen bundles of the anterior stroma. (c, d, e, f, and g) courtesy of the Institute for In Vitro Sciences.

Eye effects depend on the depth and degree of injury

Ocular irritants differ from corrosive (severely irritating) substances based upon the severity of the induced injuries. Ocular irritants induce local, reversible inflammatory reactions, whereas corrosive substances irreversibly damage ocular tissues, leading to possible vision decay or blindness [88]. Ocular changes and lesions in the anterior segment of the eye – including corneal swelling, corneal opacity, iritis, conjunctival redness and/or conjunctival chemosis – are caused following penetration of cell-damaging chemicals through the corneal and/or conjunctival epithelia, or by direct disruption of surface membranes. Since classification, which dictates precautionary measures, is based upon the potential adverse impacts, it is critical that human relevant models are utilized to make human relevant predictions.

The corneal and conjunctival epithelia provide effective barriers to penetration or permeation by most substances, provided those substances do not have the ability to denature, erode, dissociate or solubilize those tissues. It has been demonstrated that among ophthalmic drugs (which are presumably extremely mild to corneal tissues), lipophilic drugs are more likely to permeate the epithelium than hydrophilic drugs, due to the lipid-rich construction of the cell membranes within the epithelium. Whereas the epithelium is a lipophilic environment, the stroma is an aqueous rich environment that presents an effective barrier to permeation of hydrophobic drugs but readily allows permeation of hydrophilic drugs [3]. Accordingly, chemicals that are unable to penetrate the epithelium are also unlikely to cause irritation. In contrast, those chemicals that are able to disrupt the epithelial barrier by solvent diffusion through the cellular environment, injury to the tight junctions between the squamous epithelial cells, or necrotic degradation of the epithelium may also induce cytotoxic and cytolytic effects within the cornea.

Applying the depth of injury (DOI) concept is critical to understanding ocular injuries and in selecting the appropriate test methods to model them [89]. This is because injury to the cells in different regions of the eye have different impacts. In general, cell or tissue injury occurring progressively deeper into the cornea results in increasingly worse injuries. Whereas numerous attempts have been reported [45,46,74,89–92] using various histopathology methods to determine the thresholds delineating the DOI between irritation categories in *ex vivo*/*in vitro* corneal systems, not surprisingly some overlap in the DOI between categories is observed. The following summarizes generalized findings on the depth of injuries, relative to chemical irritation potentials.

Slight irritants tend only to injure cells within the superficial corneal epithelium whereas mild irritants also injure cells deeper into the epithelium. Some mild irritants may also induce injuries and changes in the uppermost stroma. Moderate irritants injure deeper into the epithelium and typically also induce injuries to the stromal keratocytes in the anterior stroma [90]. In a recent study evaluating the use of live/dead staining techniques in excised rabbit eyes to determine the DOI of 16 chemicals covering the range of EPA categories (based upon Draize test results), it was demonstrated that some but not all of the EPA category III chemicals induced keratocyte injuries which were determined to impact no deeper than the upper 10% of the stroma. In comparison, the category II materials tended to induce stromal injuries notably deeper into the stroma. It should be noted that some overlap was measured in the DOI, particularly among the category II and III chemicals [91]; however, this overlap is not unexpected given the variability of the rabbit response, particularly for chemicals that are classified as mild and moderate [18]. Epithelial permeability increases if substances injure or destroy the epithelial barrier function initially by injuring tight junctions or desmosomes and progressively by necrosis of the epithelial layers. Substances causing eye corrosion damage cells within the epithelium, deep stroma, and often, but not always, the corneal endothelium and thus irreversibly injure the cornea [3].

Whereas the depth of the injury describes which cells or tissue layers are affected, the degree of injury describes the extent of the injuries and is equally critical in eye irritation assessments [90]. For example, an overall loss of stromal keratocytes in the upper stroma will have a remarkably worse outcome on corneal recoverability relative to another injury where only a few keratocytes are lost at the same depth of injury.

The depth and the degree of injury are highly dependent upon the nature, mode of action, dose, and duration of exposures of the specific chemistries. The nature of those chemical exposures dictates the potential to penetrate into the cornea, starting superficially with the initial contact with the squamous epithelium, and progressing deeper into the cornea. Dose and chemical concentration play a significant role in whether an inherently irritant chemical induces a milder, reversible injury or induces severe irreversible damage. For example, exposures to sodium hydroxide at high concentrations ($\geq$1 N) can readily induce deep penetrating irreversible injuries into the stroma and endothelium, while low concentrations, such as those used in product formulations (e.g. to maintain a neutral pH), may have very little measurable impact on the cornea [93]. In fact, since the depth and degree of corneal injuries are progressively dependent upon dose, concentration and duration of exposure, the recoverable and irreversible apical events are part of the spectrum of ocular injury responses.

Reversibility of effects

It is essential to consider reversibility when assessing the potential effects of chemicals on the eye. As presented above, the potential for reversibility of damage can occur in a dose-dependent manner. Chemicals can trigger anti-inflammatory processes and repair mechanisms, such as recruitment of phagocytes, which work to repair cellular and tissue injury. Epithelial cells have the capacity to manage low-dose exposures to irritants – on a daily and repeated basis – without injury [94]. Chemical doses above a certain threshold have the potential to overwhelm anti-inflammatory mediators and repair mechanisms, demonstrating a delicate balance between inflammatory and anti-inflammatory processes [95].

Significant inflammation responses in the basal epithelium may trigger matrix proteases with subsequent loss or sloughing of epithelial tissues [96,97]. As a general rule, corneal and conjunctival injuries limited to the epithelial tissues are likely reversible and recoverable, while deeper injuries, especially those into the basal epithelial cells and stroma, are less likely recoverable in a meaningful timeframe.

Corneal injuries that are limited to the superficial conjunctival or corneal squamous epithelium, as well as the wing cell layer of the epithelium (through the squamous epithelium and into the wing cells of the corneal epithelium), typically recover by upward cell displacement and differentiation to re-establish the squamous epithelium. Deeper corneal injuries into the basal cell layer of the epithelium may take longer for meaningful recovery, particularly when the epithelial cell sheet is lost. In such cases of epithelial cell sheet loss, the lower epithelium recovers by both horizontal epithelial sheet migration from the outer limbal tissues, and subsequent upward cell displacement and differentiation to re-establish the epithelium. Where the corneal injuries are extensive and affect the limbal tissues, recovery may be less likely in a meaningful timeframe. Although epithelial tissue injuries are generally recoverable for certain chemistries, irreversible phenotypic changes to the basal cells, or irreversible changes to the anterior limiting lamina or basal lamina, may result in incomplete recovery, or recurrent epithelial loss and erosions. An example of the latter was observed in soldiers during World War I after battlefield exposures to the chemical warfare agent, sulphur mustard, when after initial recovery, late recurrent ulcerative episodes of the cornea were reported, presumably as a result of cross-linking and denaturation of collagen [98].

Damage into the stroma typically involves necrotic or apoptotic loss of keratocytes, and, depending both upon the nature and mode of chemical action and the depth and degree of stromal injury, may define whether the injury is reversible or non-reversible. Cytolytic injuries to keratocytes result in release of IL-1α, initiating recruit of inflammatory neutrophils from the limbal vasculature [99]. The degree of the inflammatory response is thought to be related to the number of damaged keratocytes. Stromal opacity due to loss of keratocytes limited to the upper stroma may be reversible, particularly if the extent or frequency of keratocyte loss is minimal. Shortly after cell death, keratocytes are replaced by new keratocytes through mitosis of adjacent cells [100]. Recovery is expected to take considerable time for re-establishment of the basal membrane substrate, epithelial tissue recovery, and re-establishment by keratocytes of a normal stromal collagen environment. Damage deeper into the stroma with pervasive loss of keratocytes would overwhelmingly be irreversible as significant risks of abnormal collagen deposition and fibrosis, as well as pannus and neovascularization occur with increasing loss of keratocytes, and concomitant inflammatory responses.

Damage and/or loss of the endothelial layer would almost always be associated with chemical injury superficially in the stroma and epithelium. Unlike in rabbits, the loss of endothelium in humans is not recoverable, and areas showing loss of endothelial function will result in local or diffuse stromal swelling and concomitant opacities.

The speed of recovery differs between rabbits and humans, with effects seen in rabbits more prominent than those observed in humans at comparable dose volumes [49,101,102]. Additionally, while substances are often quickly removed by the human blinking and tearing responses [1], instillation of the test substance directly into the conjunctival sac of the rabbit per the Organization for Economic Co-Operation and Development (OECD) TG contributes to the exaggerated response seen in the rabbit.

Mechanism of eye irritation

Chemicals can damage cells through multiple modes of action, including lysis of the cell membrane; promotion of coagulation or denaturation of macromolecules; solubilization of cell macromolecules; promotion of saponification of lipids; and/or covalent interaction with macromolecules [103–105]. Different *in vitro* and *ex vivo* models can be used to assess these various modes of action.

Adverse outcomes in the eye become progressively more severe with greater depth of chemical penetration and degree of toxic effects induced within each tissue layer. The adverse outcome tends to be cumulative from the initial chemical contact with the superficial outer epithelium to deep into the underlying subconjunctival scleral/tarsal tissues or corneal stromal tissues. Accordingly, the overall adverse outcomes at the organ level tend to be dictated by the prominent injuries at the deepest tissue levels. Because the adverse outcome of a toxic exposure may differ in different tissues of the eye, one can envision individual pathways outlining eye effects for each of the major corneal and conjunctival tissue layers (Figure 2).

Test methods

In addition to the *in vivo* rabbit eye test developed in 1944, multiple *in vitro* and *ex vivo* methods have been adopted by the OECD, including the reconstructed human cornea-like epithelium (RhCE) test system, the bovine corneal opacity and permeability (BCOP) test method, the isolated chicken eye (ICE) test method, the fluorescein leakage (FL) test method, the short-time exposure (STE) test method, the vitrigel-eye irritancy (EIT) test method, and the ocular irritection® macromolecular test method. There are also several methods that are not yet OECD TGs but are being used to assess ocular effects, including the isolated rabbit eye test method, neutral red release assay (NRR), OptiSafe, porcine cornea opacity/reversibility assay (PorCORA), EYEIRR-IS®, the *ex vivo* eye irritation (EVEIT) test, and cytosensor microphysiometer assay. A comprehensive list of available methods and a summary of their relevance to humans can be found in Table 1.

All of the test methods presented in Table 1 include (i) a test system or platform (the organism, tissues, cells, or biomolecular matrix to which test material is applied, and from which the relevant endpoints are determined), (ii) a dosing and exposure protocol, and (iii) the mechanistically based endpoint methods. In the development of many of the *in vitro* and *ex vivo* eye irritation tests, dose and exposure

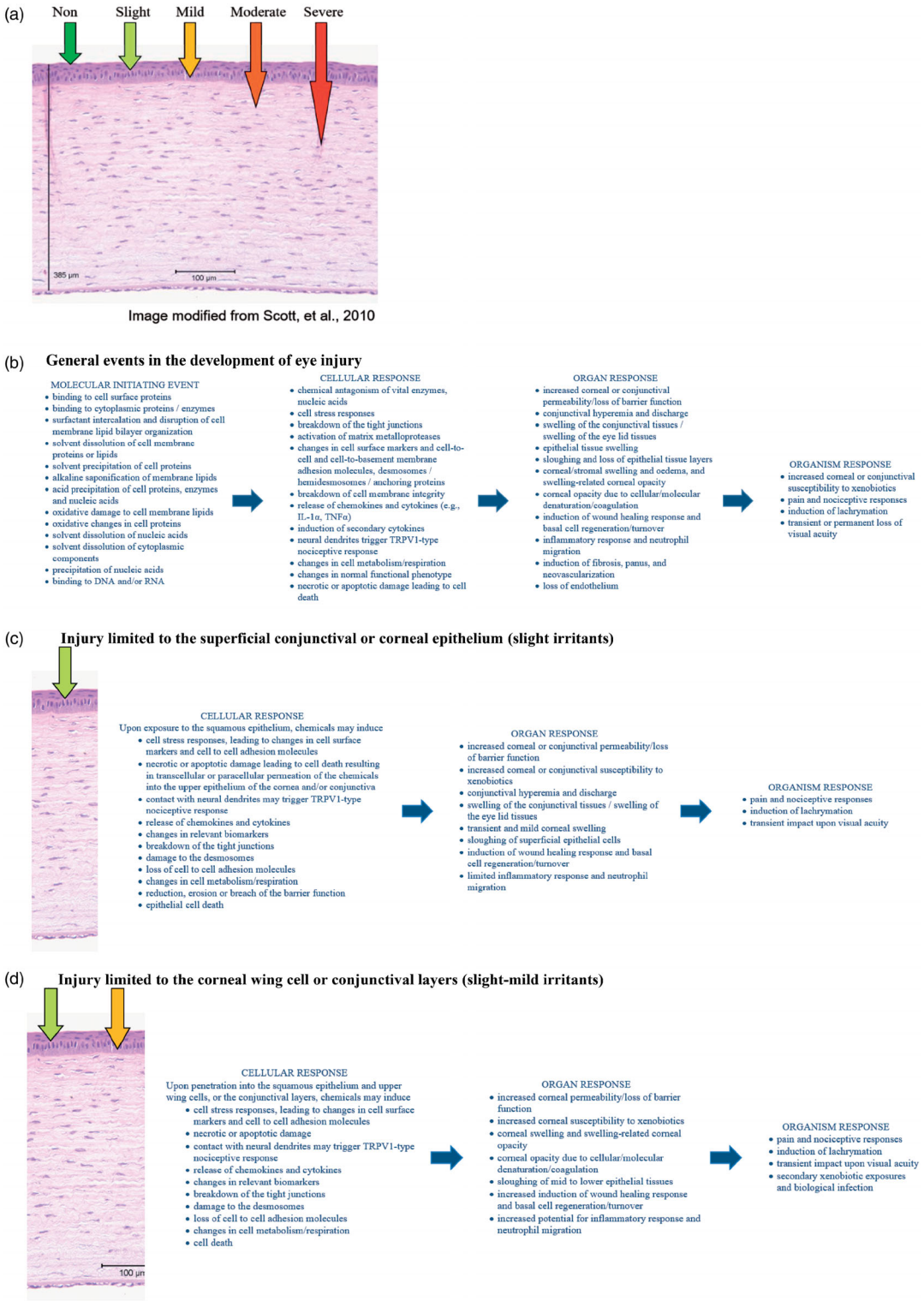

Figure 2. Mechanism of eye irritation. (a) Depth of Injury Model modified from Scott et al. [105]. The depth of injury is predictive of the degree and duration of injury. (b) Shows general events that may occur in the development of eye injury, while (c) through (g) show events relevant to specific parts of the eye. Listings are intended to comprehensively present the numerous potential responses and pathways of eye injury, rather than represent linear mappings. (c) Injury limited to the superficial conjunctival or corneal squamous epithelium. This limited injury is typically reversible, and recovers by upward cell displacement and differentiation to re-establish the squamous epithelium. (d) Injury limited to the wing cell layer of the epithelium (through the squamous epithelium and into the upper wing cells of the corneal epithelium). This limited injury is typically reversible, and recovers by upward cell displacement and differentiation to re-establish the epithelium. (e) Injury into the lower wing cell and basal cell layers of the epithelium (through the corneal epithelium into the lower wing cell and basal cell layers). This progressive injury is often reversible, but may take longer for meaningful recovery. Lower epithelium recovers by both horizontal epithelial sheet migration and upward cell displacement and differentiation to re-establish the epithelium. For some chemistries, irreversible changes to the anterior limiting lamina or basal lamina, or irreversible phenotypic changes to the basal cells may result in incomplete recovery, or recurrent epithelial loss and erosions. (f) Damage into the corneal stroma (through the corneal epithelium into the corneal stroma). Damage and loss of keratocytes in the stroma often results in moderate to severe ocular irritation, depending upon the depth and degree of stromal injury. While a moderate irritant may cause cellular damage and keratocyte loss limited to the upper stroma that could be reversible, a severe irritant (corrosive) would cause damage deeper into the stroma with pervasive loss of keratocytes that is irreversible. Recovery is expected to take considerable time for re-establishment of the basal membrane substrate, epithelial tissue recovery, and re-establishment by keratocytes of a normal stromal collagen environment. Significant risks of abnormal collagen deposition and fibrosis, as well as pannus and neovascularization occur with notable stromal damage. (g) Damage into the corneal endothelium. Damage and/or loss of the endothelial layer would almost always be associated with chemical damage superficially in the stroma and epithelium. The loss of endothelium in humans is not recoverable, and areas showing loss of endothelial function will result in local or diffuse stromal swelling and concomitant opacities.

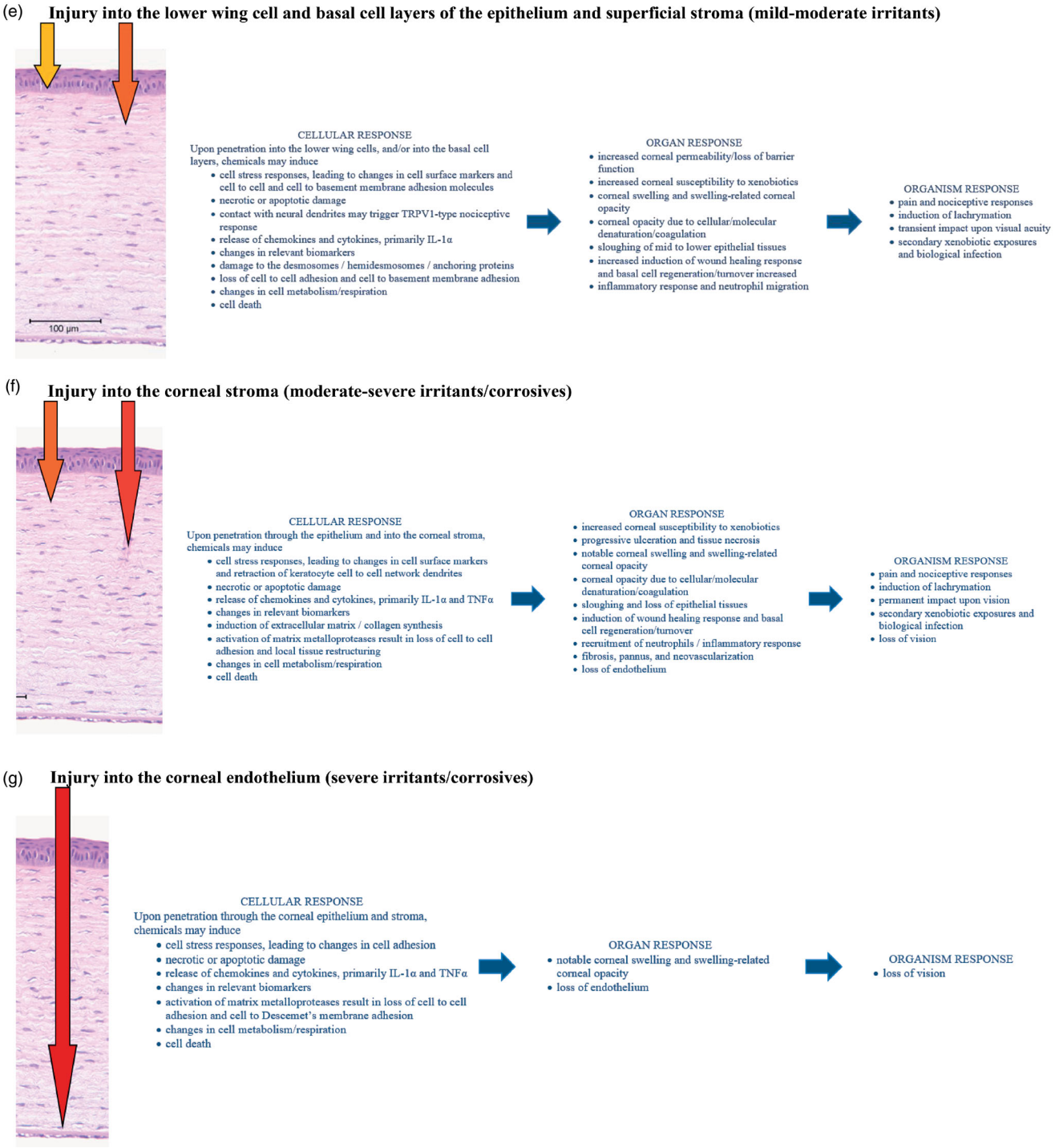

Figure 2. Continued.

protocols were optimized to their respective test systems so that the range and magnitude of the endpoint response values better fit the rabbit values or rabbit-based irritation categories. Since each of the test systems differ in structure and sensitivity, the dose volumes and exposure times differ accordingly, and do not typically reflect the rabbit *in vivo* exposure regimen (nor necessarily model a human exposure). For example, the STE and NRR assays utilize short, 5 min exposures that are intended to model splash events and they are of sufficiently short duration to allow for a range of responses to be expressed in the sensitive cell monolayer, but they do not directly reflect any specific *in vivo* exposure times. Furthermore, several *in vitro* and *ex vivo* eye irritation tests require specific protocols for testing solid materials that differ from the protocols for testing liquid materials by notably increasing exposure times or doses to better reflect the rabbit data for solid materials (arguably as a consequence of abrasive injuries in the rabbit test that are inherent with solid materials, rather than solely responding to the chemical effects). Importantly, one can exercise precise control of the

application and termination of the dose volumes and the duration of exposures to *in vitro* and *ex vivo* test systems, thereby greatly reducing a source of variability in the test results. Conversely, in the rabbit test, spilling and leakage of the applied dose from the cul-de-sac within seconds after application undermines reproducible, quantifiable measures of exposure and associated effects.

Given their complexity and composition, agrochemical formulations, which often include some combination of wetting agents, dispersants, solvents, and adjuvants, may fall outside of the applicability domains of some test methods [8]. For example, those test methods that require preparing a dilution series in an aqueous vehicle may not be suitable for testing mixtures that are not uniformly in solution, or for testing immiscible liquids or solids that are not water-soluble. Consequently, in such cases, the bioavailability of the insoluble components is called into question, and accordingly, other test methods that do not rely upon preparation of dilutions should be used.

Some methods have been incorporated into an EPA alternate testing framework for classification of eye irritation potential of EPA pesticide products that can be used for antimicrobial cleaning products and, on a case-by-case basis, for other classes of pesticides and pesticide products, including conventional, biochemical, and other antimicrobial pesticides without cleaning claims [32]. The methods are also included in an OECD guidance document on an integrated approach on testing and assessment (IATA) for serious eye damage and eye irritation that describes how physical chemical properties and existing or generated data can be integrated and used for decision making [1].

Discussion

Any of the *in vitro* and *ex vivo* methods identified in Table 1 as applicable to a bottom-up approach have been shown to be useful for identifying minimal or non-irritants, provided that those ingredients and formulations are compatible with the test systems (Figure 3). Considering the structure of the human eye and the mechanistic pathways leading to chemically induced adverse effects in the eye, 3D reconstructed human corneal tissues and *ex vivo* corneal tissues (e.g. BCOP and ICE) are particularly useful models for evaluating the spectrum of eye irritation in humans. These models have the ability to most closely recapitulate certain key events in the pathways to eye irritation at the cellular (3D reconstructed human corneal tissues and *ex vivo* corneal tissues) and organ levels (*ex vivo* corneal tissues). These systems also offer the advantage of precise and well-controlled exposure protocols that increase the reliability and reproducibility of the results.

3D reconstructed human corneal tissues and *ex vivo* corneal tissues model the exposure and permeation kinetics of chemicals from the sites of exposure at the outermost squamous epithelium deeper into the corneal model. Only full thickness models that include the different barrier properties from corneal epithelium to endothelium fully model the potential penetration kinetics of a chemical.

The 3D reconstructed human corneal tissue models are particularly appealing because they measure a critical event in the pathway to irritation – cytotoxicity – within a stratified construct of cells from the species of interest. These tissue models can also be used to determine chemical-induced cytokine release and expression, providing further relevance to the cascade of events associated with eye irritation. Since the key adverse events typically associated with mild to moderate irritants occur within the epithelial layers, they can be used to distinguish between irritants and non-irritants and, because of the stratified epithelial construct, can provide some information about DOI within the corneal epithelium. The addition of a stromal layer would allow for further discrimination between mild and moderate irritants through DOI measurements, and more importantly provide discrimination between mild or moderate irritants and severe irritants (corrosives). Future consideration could be given to the addition of an organized stromal matrix containing stromal cells and a functional endothelium, which would further enhance the relevance of these 3D reconstructed human tissue models, allowing them to thoroughly provide corneal DOI information to better predict reversibility. Lastly, having the ability to evaluate the degree of cytotoxicity, as well as other initial key events such as cytokine release, will improve the ability to predict the severity of corneal injuries and the likelihood for meaningful recovery. Importantly, however, adapted protocols using RhCE models without a stromal or endothelial layer are currently under review at the OECD and may provide sufficient information to discriminate between severe irritants (corrosives) and moderate irritants.

Ex vivo corneal tissues provide a full thickness model (i.e. epithelial, stromal, and endothelial layers) and, therefore, when including endpoints such as histopathology can provide detailed information about DOI. Since the key adverse events typically associated with moderate to severe eye irritants (corrosives) occur within the stroma, these full thickness models uniquely provide the specific cells and structures of the stroma to model chemical permeation to the target cells, and with the inclusion of histopathology, they provide a mechanistic basis for differentiating between types of recoverable and irreversible corneal injuries. Even though there may be interspecies differences due to the use of non-human cells, the relevance of these non-human *ex vivo* models is justified in most cases where common chemical mechanisms of action inducing cell cytotoxic responses are observed across species.

Consistent with the OECD IATA, combining test results with information specific to the test substance (e.g. physical chemical properties) can be used to better address the range of key events that distinguish severe (corrosive), moderate, mild, or non-irritants, and to inform on the likelihood of recovery in humans. Reversibility predictions may be supplemented by utilizing *in silico* tools to identify those chemistries (such as protein denaturing/precipitating chemistries, reactive chemistries, alkylating agents) likely to induce irreversible phenotypic changes to the basal or limbal cells, or induce irreversible changes to the Bowman's layer or basal lamina, without necessarily involving the stromal or endothelial elements. Furthermore, consideration of information from

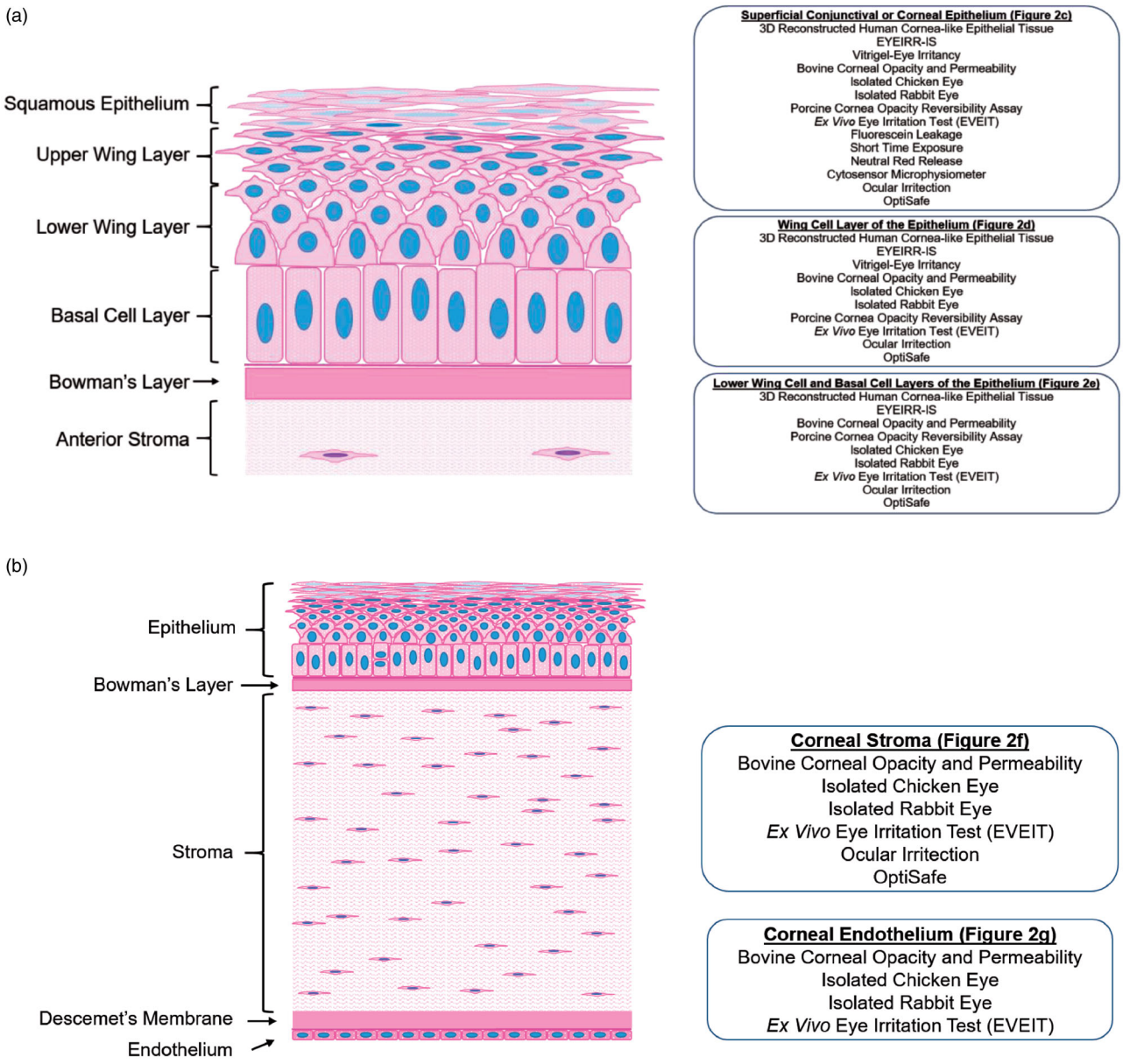

Figure 3. Schematic of a human corneal section showing which *in vitro/ex vivo* assays are appropriate for evaluating specific layers, with models relevant to the (a) corneal epithelium or (b) full thickness cornea.

other endpoints can inform toxicity predictions; for example, knowing that a chemical is highly irritating to the skin or respiratory tract as a result of a cytotoxic mechanism may suggest that it is likely irritating to the eye as well. Results from multiple test methods may be combined – while conducting multiple tests instead of a single *in vivo* rabbit eye irritation test may be more costly in the short-term, they should produce more human-relevant results (and therefore avoid costs that would be associated with having to pull a harmful product from the market, or loss of market share due to irrelevant over-predictions) and the cost of these assays may decrease over time as they are more widely implemented. In addition, while *in silico* assessment of mixtures proves challenging, partnerships with industry could be leveraged to provide data for Quantitative Structure Activity Relationship (QSAR) model building for agrochemical formulations.

Various methods, alone or in combination, may be appropriate for use, depending on the circumstances; therefore, scientific justification for using a certain approach should be communicated to the regulatory agency upon submission (Figure 4). Thus, there is not a single proposed testing strategy, rather various approaches may be acceptable when based on the properties of the test substance, the purpose of testing, existing data, and human-relevant *in vitro/ex vivo* test methods.

Conclusions

A component of the EPA Administrator's 2019 directive to staff to reduce the requests for and funding of animal studies by 2035 was the request to develop a work plan that addresses the subject of "validation to ensure that NAMs [new approach methods] are equivalent to or better than the

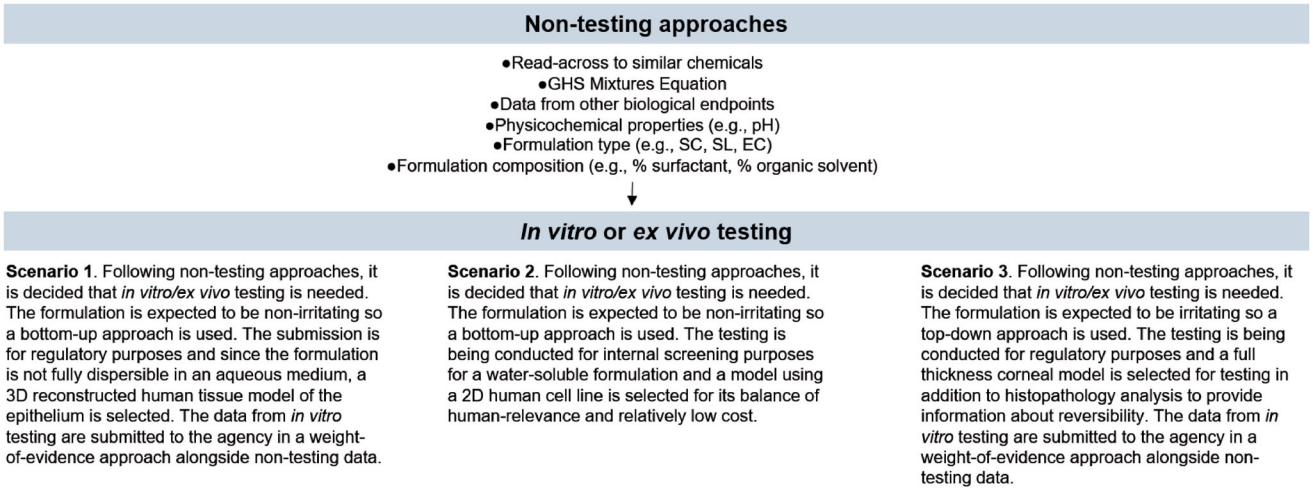

Figure 4. Example scenarios. There are many considerations and variables that drive the "best" test methods and testing strategy selected for each scenario. For example, the purpose of testing must be considered e.g. hazard versus risk evaluations, worker versus consumer safety, regulatory testing requirement versus product development. In addition, the type of chemical must be considered e.g. neat chemicals versus mixtures of chemicals, as well as their natures, formulations, and expected irritation levels. Practical factors must also be considered e.g. budget, timing, method availability, availability of test material-specific reference data per method, and historical data. To derive the most appropriate testing plan, a researcher should work internally or with their contract research organization to apply the best science and develop appropriate approaches. Based on integration and analysis of the information provided in this paper, the following general scenarios describe possible approaches for identifying an agrochemical formulation's potential to cause eye irritation. GHS: Globally Harmonized System; SC: suspension concentrate; SL: soluble concentrate; EC: emulsifiable concentrate; 3D: three-dimensional; 2D: two-dimensional. GHS mixtures equation [106].

animal tests replaced" [107]. This work plan was released in June 2020 [108]. Similar language is present in the amended Toxic Substances Control Act (TSCA) that requires that NAMs provide "information of equivalent or better scientific quality and relevance" than traditional animal tests. A key consideration in the evaluation of what it means to be "equal to or better" is the explicit consideration of the variability, human relevance, and predictivity of the animal data. This paper is designed to consider these factors for eye irritation.

The rabbit eye test is widely used to meet regulatory testing requirements for agrochemical formulations, and has been a requirement for pesticide registration for decades [109]. However, the rabbit test involves subjective examination of ocular lesions, uses an exaggerated exposure duration, provides limited mechanistic information, has never been validated for its relevance to humans, uses live animals, and is associated with considerable variability (see Table 1 for details). Furthermore, interspecies differences in structure, anatomy, and physiology exist between rabbit and human eyes, for example, rabbits have a nictitating membrane, higher pH of the eye, a larger conjunctival cul-de-sac, and are not as efficient in tear production. Thus, the rabbit test is not appropriate for use as a standard for evaluating new methods.

The *in vitro* and *ex vivo* models described herein are more human relevant than the rabbit test because they include one or more of the following properties: (a) they allow for more precise control of the application and termination of dosing, (b) they model corneal tissue barrier functions and penetration kinetics, (c) they include relevant cell types within each of the tissue layers, (d) they provide quantitative results, (e) they have been shown to be reproducible and repeatable, (f) they do not directly use live animals for testing, and/or (g) they discriminate a range of cytotoxic responses within each layer. It is not necessary for a test system to include all of these traits to be useful and relevant; a model with any one of these characteristics could be useful to address specific events in the assessment of eye irritation. Considering all available information, the *in vitro/ex vivo* methods presented in this paper are equivalent or scientifically superior to the use of the rabbit test.

Where discordant results exist between NAMs and the rabbit test, findings from the *in vitro* and *ex vivo* systems described herein should carry more weight than the rabbit data. The scientific validity of an *in vitro/ex vivo* method should be assessed by understanding the assay's relevance to human biology and mechanisms of eye irritation. Ultimately, a replacement method that provides a model grounded in human biology will be as good as or better than the currently used rabbit test at protecting human health.

Next steps

A careful consideration of the benefits and the limitations of *in vitro* and *ex vivo* eye tests show that they can be used today to make human-relevant regulatory decisions. There are also steps that could be taken to further improve existing models, including:

- Improve existing 3D reconstructed human corneal models to create a full thickness corneal model, complete with stromal and endothelial cells. Such a human cell-based method that recapitulates the distinct layers of the human cornea and provides information on the DOI following chemical exposure could be effective at distinguishing moderate and severe irritants (corrosives) and predicting reversibility [110]. Since cell cytotoxicity is an early key event in eye irritation, and related to downstream consequences depending upon the tissue layers affected, the model should be able to assess cytotoxic

effects throughout the full thickness structure [111]. Furthermore, advanced models of a blinking human eye may be more sophisticated than needed to predict irritancy, but could provide useful information and incorporate effects of mechanical injury resulting from blinking that are not captured in other *in vitro* tests [112]. Additional investigations could also include use of optical coherence tomography to assess full thickness injury in a non-invasive way, as is done in the EVEIT test.

- Move away from high dose, longer chemical exposures in favour of more human-relevant risk-based exposure scenarios. This will better align test exposures with the common human scenario, where a substance is mostly expelled from the eye in a matter of minutes following exposure. Furthermore, multiple exposure scenarios can be more easily tested in *in vitro* and *ex vivo* systems, allowing for the assessment of a splash scenario followed by immediate rinsing as well as an intermittent exposure over a full workday.
- Establish an acceptable level of variability for assays. Despite the recognized variability of the rabbit test [18], particularly in the mild and moderate irritancy range, it has been considered sufficient for regulatory use and thus an acceptable level of confidence in such results has been conveyed. The level of confidence/variability deemed acceptable based on historical use of the rabbit test, in combination with the information about human biology and mechanisms described in this paper, can be used to evaluate the validity of a method. Similar analyses have been proposed to define acceptable performance of NAMs for other endpoints [113–115]. Ultimately, this permits the level of variability for a new method to be held to a similar standard as that for the animal test.
- Re-evaluate existing data considering the conclusions and recommendations in this paper. The US National Toxicology Program (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM), EPA, the PETA Science Consortium International e.V., and a group of agrochemical companies have collaborated to conduct both retrospective and prospective assessments of available *in vitro* and *ex vivo* methods to determine their usefulness and limitations for eye irritation testing of agrochemical formulations. The prospective testing results and associated analyses have been presented [13]. These results provide an opportunity to consider how best to apply these methods in regulatory decision making based on the discussions in this paper. Accordingly, additional analyses are ongoing that are intended to develop a framework for doing so.
- Generate additional *in vitro/ex vivo* data where needed for agrochemical formulations. While there are substantial existing data for many of the *in vitro* and *ex vivo* assays (e.g. BCOP, ICE, NRR, and EpiOcular™), there are assays for which there remain limited data for agrochemical formulations. In particular, because of its use of a 3D human tissue model, additional information about the applicability of the EYEIRR-IS® assay to agrochemical formulations would be useful. In addition, since agrochemical formulations often target very specific or unique cellular processes, the genomic-based platform might reveal unique adverse outcome pathways that other test methods do not address. Transparency in the model and its associated decision criteria are essential for regulatory utility. Importantly, any future or existing *in vitro/ex vivo* data need to be vetted for their relevance to human biology and not to direct alignment with hazard categories derived based on rabbit data.

The above steps reiterate that opportunities to further refine existing *in vitro* and *ex vivo* models can be explored while they are being used today for decision-making. Overall, considering the variability of the currently used rabbit test and an understanding of human biology and mechanisms of eye irritation presented in this paper, to best protect human health, data from the *in vitro/ex vivo* methods are considered applicable for use at this time.

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Disclaimer

The views expressed in this paper are those of the authors and do not necessarily represent the views or the policies of the US Environmental Protection Agency or specifically of EPA's Office of Pesticide Programs

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